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Polyclonal B Cell Responses to Conserved Neutralization Epitopes in a Subset of HIV-1-Infected Individuals▽†

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A small proportion of HIV-infected individuals generate a neutralizing antibody (NAb) response of exceptional magnitude and breadth. A detailed analysis of the critical epitopes targeted by broadly neutralizing antibodies should help to define optimal targets for vaccine design. HIV-1-infected subjects with potent cross-reactive serum neutralizing antibodies were identified by assaying sera from 308 subjects against a multiclade panel of 12 "tier 2" viruses (4 each of subtypes A, B, and C). Various neutralizing epitope specificities were determined for the top 9 neutralizers, including clade A-, clade B-, clade C-, and clade A/C-infected donors, by using a comprehensive set of assays. In some subjects, neutralization breadth was mediated by two or more antibody specificities. Although antibodies to the gp41 membrane-proximal external region (MPER) were identified in some subjects, the subjects with the greatest neutralization breadth targeted gp120 epitopes, including the CD4 binding site, a glycan-containing quaternary epitope formed by the V2 and V3 loops, or an outer domain epitope containing a glycan at residue N332. The broadly reactive HIV-1 neutralization observed in some subjects is mediated by antibodies targeting several conserved regions on the HIV-1 envelope glycoprotein.

Broadly neutralizing antibodies (bnAbs) protect nonhuman primates from experimental simian-human immunodeficiency virus (SHIV) challenge (46–48, 61, 64, 81, 108) and are widely expected to be a key component of protective immunity conferred by an effective human immunodeficiency virus type 1 (HIV-1) vaccine (63). HIV-1 neutralization depends on the ability of Abs to recognize native envelope glycoprotein (Env) spikes, which consist of three surface gp120 subunits noncovalently linked to three membrane-spanning gp41 subunits (32, 70, 91). These spikes are compact structures protected by a heavy glycan shield that disfavors nAb recognition. Not all nAb epitopes are shielded, and in fact, the extent of shielding differs considerably among strains. Thus, some viruses are highly sensitive to neutralization by heterologous sera from HIV-1-infected individuals and are classified as having a tier 1 neutralization phenotype. However, most circulating strains are considerably less sensitive to heterologous neutralization and are classified as having a tier 2 phenotype (63).

A potent autologous nAb response against the early infecting virus is generally seen within the first year of HIV-1 infection (62, 83, 104, 113). Longitudinal studies have revealed that the virus and the host nAb response continually evolve (11, 73, 83, 86, 113). After several years of infection, an estimated 10 to 30% of individuals develop bnAb responses (4, 7, 28, 52, 57, 66, 72, 88, 89). Although these extraordinary responses have little impact in the setting of established infection, they could have a major impact on transmission if they could be induced by vaccines prior to virus exposure. Thus, the epitopes and biological processes that give rise to these exceptional bnAb responses are of considerable interest for rational vaccine design (114).

Until recently, only 4 monoclonal Abs (MAbs) (2G12, b12, 2F5, and 4E10), all from HIV-1 clade B-infected individuals, were known to potently neutralize genetically diverse isolates of HIV-1

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(8, 13, 75, 107, 119). MAb 2G12 recognizes a tight cluster of glycans on the gp120 silent domain (14, 87, 93, 107). MAb b12 binds an epitope that overlaps the CD4 binding site (CD4bs) of gp120 (13). MAbs 2F5 and 4E10 (75, 119) recognize adjacent epitopes in the membrane-proximal external region (MPER) of gp41. Three of these MAbs (b12, 2G12, and 2F5) exhibit limited breadth and/or potency against non-clade B viruses, which account for the majority of infections worldwide (13). MAb 4E10 neutralizes more broadly, albeit usually at a lower magnitude. Other MAbs, including those directed to the V3 loop, usually exhibit a more limited profile of neutralization (51, 80), while MAbs directed to CD4-induced epitopes usually fail to neutralize tier 2 viruses altogether (8, 53, 115).

To date, vaccine immunogens targeting these MAb epitopes have not succeeded in eliciting bnAbs (42, 69, 82, 92). Consequently, there has been a resurgence of interest in isolating new bnMAbs, particularly from non-clade B-infected individuals, in the hope that these new epitopes will be more amenable to vaccine development (9, 12, 16, 18, 31, 95). Several new MAbs, most notably VRC01, VRC02, VRC03, PG9, PG16, and HJ16 (18, 111, 114, 118), each of which possesses potent activity against multiple genetic subtypes of the virus, have been identified.

These new MAbs were derived from donors of diverse geographic backgrounds infected with one of three different HIV-1 subtypes: A (MAbs PG9 and PG16), B (VRC01, VRC02, and VRC03), and C (HJ16). This emphasizes the importance of including globally diverse HIV-1-infected donor material in bnAb epitope discovery efforts (76, 102). MAbs VRC01 and HJ16 recognize epitopes that overlap the CD4 binding site (18, 114), while PG9 and PG16 recognize novel quaternary epitopes involving the V1V2 and V3 loops of gp120 (27, 111). Mutations that remove sequons at positions N156 and N160 in the V2 loop eliminate the binding of PG9 and PG16, as does enzymatic deglycosylation, suggesting that glycans are involved in these quaternary epitopes. Soluble CD4 (sCD4) ablates the ability of the latter two MAbs to bind trimers, suggesting that the conformational changes alter the quaternary epitope. These V2-V3 epitopes were somewhat surprising, considering that until recently, these loops were thought to protect underlying conserved epitopes and were considered too variable to be targets of broad neutralization. These findings suggest that despite the variability of the V-loops, conserved elements that can be targets of bnAb responses do exist.

Various technologies have been used to identify the epitopes of bnAbs (reviewed in references 1, 4, 5, 7, 21, 24–26, 37, 39–41, 57, 89, 101, and 117). An analysis of serum samples from approximately 200 individuals chronically infected with HIV-1 revealed that about one-third exhibit low titers of MPER nAbs, as detected by a sensitive HIV-2/HIV-1 MPER chimera assay in which the MPER is spontaneously exposed (3). However, in all but exceptional cases, these MPER nAbs do not neutralize circulating strains of HIV-1. In most cases, MPER nAbs were ascribed to 4E10-like epitopes, but 2F5-like nAbs have also been reported in rare cases (7, 39, 41, 89, 101).

Some studies suggest that bnAbs in chronic HIV-1 infection may be oligoclonal (2, 6, 23, 43, 95, 111). Indeed, recent work suggests that the responses are limited in specificity, often resembling PG9 and PG16 (111), are restricted in their IgG heavy and light chain usage, and require extensive somatic mutation (111). Furthermore, the observation that MAbs PG9 and PG16 largely recapitulate the neutralizing activity of the donor plasma provides evidence that broad neutralization can occur in some cases via a single specificity or multiple overlapping specificities (111). An overarching question from these studies concerns the number of nAb specificities that typically mediate broad plasma neutralization (72, 90, 95, 112). Here, to gain more insights into the frequency and titer of existing bnAb specificities and possibly to identify new bnAb targets, we analyzed the specificities of plasma samples from 9 HIV-1-infected subjects who exhibited high-titer bnAbs. Distinct bnAb specificities were present in the plasma from each subject, and in some cases, more than one specificity contributed to neutralization breadth.

MATERIALS AND METHODS

Chronically HIV-1 infected subjects. A total of 308 subjects chronically infected with HIV-1 strains of clade A, B, or C were enrolled in the Center for HIV/AIDS Vaccine Immunology cohort CHAVI 001 or CHAVI 008, and plasma and serum samples (and, in some cases, purified IgG from cervical lavage [CVL]) were examined for antibody specificities and neutralizing activity. Donor enrollment was based on standard clinical diagnosis of HIV-1 infection, at which time subjects were all antiretroviral naïve. The donors were enrolled at clinical sites in Tanzania, South Africa, Malawi, and the United Kingdom. This study was approved by the institutional review boards at each of the participating institutions where samples were received or processed for end user analysis.

MAbs and sCD4. A panel of HIV-1 Env-specific MAbs (b12, VRC01, and VRC03, directed to epitopes overlapping the CD4bs of gp120 [13, 114]; 2G12, directed to a unique glycan-dependent epitope on gp120 [87, 93]; E51, directed to a CD4-inducible [CD4i] epitope on gp120 [115]; 447-52D, F425, F2A3, and 39F, directed to the gp120 V3 loop [21, 80, 96, 99]; 2F5, Z13e1, and 4E10, directed to the gp41 MPER [77, 120]; and PG9 and PG16, which recognize quaternary epitopes involving the V1, V2, and V3 loops [111]) were utilized as positive controls. Recombinant soluble CD4 (sCD4) consisting of all of the 4 outer domains was purchased from Progenics Pharmaceuticals (Tarrytown, NY).

HIV-1, HIV-2, and SIV isolates and pseudoviruses. The molecularly cloned Env-pseudotyped viruses used for neutralization and mapping experiments were produced by cotransfection of 293T cells with an Env-expressing plasmid and one of two Env-deficient HIV-1 genomic backbone plasmids: pNL-LucR-E (for CF2 neutralization assays) or pSG3ΔEnv (for TZM-bl neutralization assays) (5, 55, 56). Env plasmids included subtype B and C tier 2 reference strains (55, 56) and other strains described previously (5, 57, 97, 98). SHIV-89.6P and SHIV-SF162P3 were uncloned live viruses grown in human peripheral blood mononuclear cells (PBMCs) (44). A series of point mutants was generated in the JR-FL SOS, ConC, and CAP45.G3 Env plasmids. Plasmids expressing tier 1 Envs SF162.LS and MW965.26 (15) were obtained from L. Stamatatos (Seattle Biomedical Research Institute) (103) and the NIH AIDS Reference and Reagent Program (originally provided by B. Hahn) (33), respectively. Chimeric HIV-2 Envs containing various segments of the HIV-1 MPER were constructed from a parental HIV-2/7312A plasmid (3, 39). The SIVmac239CS.23 Env plasmid was subcloned from an animal challenge stock of molecularly cloned SIVmac239/nef-open provided by R. Desrosiers (New England Primate Center).

HIV-1 Env IgG subclass binding antibody assay. IgG subclasses were determined by a custom HIV-1 Luminex assay with modifications for specific IgG subclasses (105). Briefly, a total of 5×10^6 carboxylated fluorescent beads (Luminex Corp., Austin, TX) were covalently coupled to 25 μg of one of the purified HIV antigens and were incubated with plasma or CVL samples. HIV-specific antibody isotypes were detected with goat anti-human IgA (Jackson ImmunoResearch, West Grove, PA), mouse anti-human IgG1 (BD Pharmingen), mouse anti-human IgG2 (Southern Biotech), mouse anti-human IgG3 (Calbiochem), or mouse anti-human IgG4 (BD Pharmingen) conjugated to biotin, at 4 μg/ml, followed by washing and incubation with streptavidin-phycoerythrin (PE) (BD Pharmingen). Beads were then washed and acquired on a Bio-Plex instrument (Bio-Rad, Hercules, CA). An HIV-1^+ plasma titration was utilized as a positive control in every assay. Background values (beads in the absence of the detection antibody) and normal human plasma were utilized as negative controls. IgG1 to IgG4 equivalents were determined by titrating purified IgG1 to IgG4 human myeloma proteins (Sigma) on beads coupled to purified

anti-human IgG1 to IgG4 antibodies, detecting with goat-anti human κ chain (Southern Biotech) conjugated to biotin, washing, and incubating with streptavidin-PE (BD Pharmingen). Binding antibody concentration (μg/ml) equivalents were calculated using a 4-parameter logistic (4PL) curve analysis.

Peptide array. Purified IgG was examined by a peptide microarray using an Env peptide library containing 15-mer peptides, overlapping by 12 amino acids, that cover the full length of the consensus HIV-1 Env gp160 sequences for subtypes A, B, C, and D, group M, circulating recombinant form 1 (CRF 1), and CRF 2 (designed by B. Korber, Los Alamos National Laboratory). The peptides were printed onto 3D-Epoxy glass slides (PolyAn GmbH, Germany) by JPT Peptide Technologies GmbH (Germany) and were analyzed with a GenePix 4000B scanner. Samples were hybridized to the library using a Tecan HS4000 hybridization workstation, followed by incubation with DyLight 649-labeled goat anti-human IgG. Fluorescence intensity was measured using a GenePix 4000B scanner and was analyzed with GenePix software.

Autoantibodies. Antibodies reactive to SSA/Ro, SS-B/La, Sm, ribonucleoprotein (RNP), Jo-1, double-stranded DNA (dsDNA), centromer B, and histone were measured by the FDA-approved AtheNA MultiLyte ANA II test kit from Zeus Scientific, Inc., according to the manufacturer's instructions (45).

Neutralization assays. Neutralization was assessed in either TZM-bl or CF2 target cells. The single-cycle TZM-bl neutralization assay was used as described previously (55). To determine the neutralizing IC_{50} (dilution inhibiting 50% of virus activity) of plasma, serially diluted heat-inactivated plasma or MAb was incubated with virus, and the neutralization dose-response curves were fit by nonlinear regression using a 4-parameter Hill slope equation. In some cases, plasma and mucosal IgG were purified for neutralization assays using protein G columns (MultiTrap plates; GE, Inc.).

The stage of HIV-1 entry inhibition targeted by nAbs was determined by using canine CF2 cell lines in standard and post-CD4/CCR5 assay formats as described previously (5, 21). Briefly, in the standard format, the virus was incubated with graded dilutions of the Ab for 1 h at 37°C. The mixture was added to CF2 cells, spinoculated, and incubated for 2 h at 37°C, after which time the medium was changed. After 3 days of culture, luciferase activity was measured. Neutralization that occurred post-CD4/CCR5 binding was measured by using mutant particles containing a disulfide bond (SOS) between gp120 and gp41 that blocks fusion (1, 5). Particles were allowed to attach to target cells for 2 h, after which time unbound particles were removed by washing, and graded concentrations of Abs were added for 1 h. Infection was activated by using 5 mM dithiothreitol (DTT) for 10 min to reduce the gp120–gp41 disulfide bond, allowing infection to proceed. When neutralization was assessed against Env point mutant viruses for mapping experiments, plasma samples were titrated in 2-fold dilutions against both the parent and the mutant in duplicate at least twice. A positive knockdown of neutralization was recorded if there was at least a 2-fold drop in the neutralizing titer. Similarly, enhancement was recorded if there was at least a 2-fold increase in the titer. A murine leukemia virus (MLV)-enveloped pseudovirus was used as a negative control in assays to confirm the absence of antiretroviral drugs, which can score positive in the assays and be mistaken for nAbs.

Plasma fractionation using paramagnetic-bead-immobilized gp120 and MPER peptides. Plasma fractionation by use of paramagnetic-bead-immobilized gp120 has been described previously (37, 41, 58). The depletion of anti-gp120 (ConC) antibodies was evaluated by an enzyme-linked immunosorbent assay (ELISA) as described elsewhere (58). Similarly, plasma was depleted of anti-MPER antibodies by using streptavidin-coated magnetic beads coated with a biotinylated linear MPER peptide (the MPR.03 peptide [KKKNEQELLELDK WASLWNWFDITNWLWYIRKKK-biotin-NH_2]) as described previously (37). The percentage of gp120-directed activity was calculated as $[(a - b)/a] \times 100$, and CD4bs activity as a percentage of total neutralization was calculated as $[(c - b)/a] \times 100$, where a stands for adsorptions against a blank, b stands for adsorptions against wild-type gp120, and c stands for adsorptions against the gp120 D368R mutant.

Native PAGE. Blue native polyacrylamide gel electrophoresis (BN-PAGE) band shifts were used to analyze Ab binding to native, virion-derived Env trimers as described previously (19–21, 70). Trimer binding was measured via depletion of the unliganded trimer using UN-SCAN-IT densitometry software (Silk Scientific) (19). Briefly, ID_{50}s were calculated with reference to control lanes in each gel, in which the density of a uncomplexed control trimer band is taken as 100% and the density of a band that is fully liganded by a nAb (either b12, 2G12, or a high concentration of plasma) is taken as 0%. Experiments were run a minimum of 3 times to confirm the accuracy of ID_{50} estimates.

ELISA. JR-FL gp120 (a gift from Progenics Pharmaceuticals) was used to coat wells of ELISA plates at 5 μg/ml. Wells were blocked with 2% nonfat milk–phosphate-buffered saline (PBS). For 2G12 competition ELISAs, MAb 2G12 or plasma was added to wells at a fixed concentration or dilution. Graded dilutions of biotinylated 2G12 were then overlaid, and alkaline phosphatase-conjugated streptavidin was used to detect binding. The signal was developed with *p*-nitrophenol and was read at 405 nM.

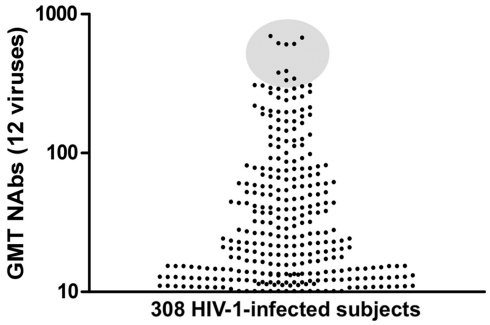

FIG. 1. Geometric mean titers (GMT) of nAb in plasma against a 12-virus panel. Plasma samples from 308 HIV-1-infected individuals from the CHAVI 001 and CHAVI 008 cohorts were assayed against 12 reference strains of subtypes B and C. GMT are plotted on a log scale (as the reciprocal plasma dilutions), and the 9 plasma samples (top 3%) with the highest titers are indicated by shading.

RESULTS

Identification and classification of broadly neutralizing plasma samples. Plasma samples from 308 chronically infected subjects enrolled in CHAVI 001 or CHAVI 008 (17, 65, 76) were assayed for neutralizing activity against 6 subtype B and 6 subtype C standard tier 2 reference strains of HIV-1 (55, 56). The results revealed a wide range of geometric mean titers (GMT) (Fig. 1). We categorized neutralization breadth into 3 "levels": "level 1" plasma samples neutralized 8 out of 12 viruses with ID_{50}s of >100; "level 2" plasma samples neutralized 10 out of 12 viruses with ID_{50}s of >100 and 8/12 viruses at >200; and "level 3" plasma samples neutralized 10/12 viruses at >200 and 8/12 viruses at >300.

Ten percent of these plasma samples were classified as level 1, 2.5% as level 2, and 1% as level 3. We selected the 9 plasma samples with the highest GMT (the top 3%), including all 3 of the level 3 plasma samples (C1-0219, C1-0457, and C1-0763) and 6 level 2 plasma samples, for mapping studies. These subjects were from diverse geographic locations in Africa and the United Kingdom and were infected with viruses derived from subtypes A, B, C, or a recombinant A/C (Table 1). The viral loads and CD4 counts at the time of sampling were typical of chronic infections, with the exception of donor C1-0440, who exhibited a low viral set point and a low CD4 count.

The plasma samples selected were further screened in neutralization assays with a panel of 26 subtype A, B, and C tier 2 viruses (Fig. 2). Although all plasma samples neutralized each clade, subtype preferences were observed, as evidenced by differences in average titers. This observation reflects similar observations from other cohorts (7, 36, 66). Plasma sample C1-0219 had the strongest nAb response against several subtype-matched A viruses, while some non-subtype A-infected subjects (C1-0440, C1-0269, C1-0534, and C8-0258) neutralized clade A viruses relatively weakly. Furthermore, two subtype-matched combinations exhibited exceptionally high titers, with ID_{50}s of >10,000 (C1-0534 neutralization of CAP45.G3 and C1-0763 neutralization of Q23.17).

TABLE 1. Clinical data for the 9 HIV-1-infected individuals with broadly cross-neutralizing plasma antibodies

Plasma sample[a]	Patient or virus characteristic					
	Country	Gender	Age (yr)	Viral load set point (RNA copies/ml)	Latest CD4 count (cells/μl)	Clade
C1-0440	Malawi	Female	22	6,402	96	C
C1-0269	Malawi	Female	36	26,125	265	C
C1-0534	South Africa	Female	29	140,601	348	C
C1-0175	Tanzania	Female	24	108,353	877	A
C1-0219*	Tanzania	Female	48	20,888	309	A
C1-0457*	Tanzania	Female	33	36,955	442	C
C1-0536	Tanzania	Female	33	50,926	299	A/C
C1-0763*	Tanzania	Female	31	390,322	570	A
C8-0258	United Kingdom	Male	47	NA[b]	772	B

[a] Asterisks indicate level 3 neutralizers.
[b] NA, not available.

To verify that plasma neutralization was IgG mediated, purified plasma IgG was tested in neutralization assays against a panel of 12 tier 2 viruses, including 4 each of subtypes A, B, and C. The neutralizing activity of the unfractionated plasma samples corresponded to the purified IgG fraction of the plasma, confirming that the neutralization was antibody mediated (see Fig. S1A in the supplemental material). Since IgG1 was the predominant subclass in plasma, we examined a multisubtype breadth panel of HIV-1 gp140s for binding (see Fig. S1B in the supplemental material). There was considerable binding antibody breadth to clade A, B, and C HIV-1 Envs, as indicated by the mean concentration (μg/ml) of anti-Env IgG1 (with the strongest binding to subtype C Envs). The linear-epitope specificities of the purified plasma IgG were tested by using peptide arrays that spanned the entire gp160 of all major subtypes and CRFs of HIV-1 (see Fig. S1C in the supplemental material). Most plasma samples bound peptides in V3, the C5 immunodominant region (79), and the gp41 immunodominant region, which are common specificities in chronic infection. Other specificities included the V1V2 loop and the N terminus and transmembrane regions of gp41. Thus, numerous multiclade binding specificities are present in broadly neutralizing HIV-1-infected donor plasma samples.

We also asked whether neutralizing activity could be detected in mucosal specimens from these donors. Epitope mapping of the purified mucosal IgG from a CVL sample from subject C1-0536 is shown in Fig. S1D in the supplemental material. Binding specificities of IgGs from CVL specimens were generally parallel to the serum IgG response, with dis-

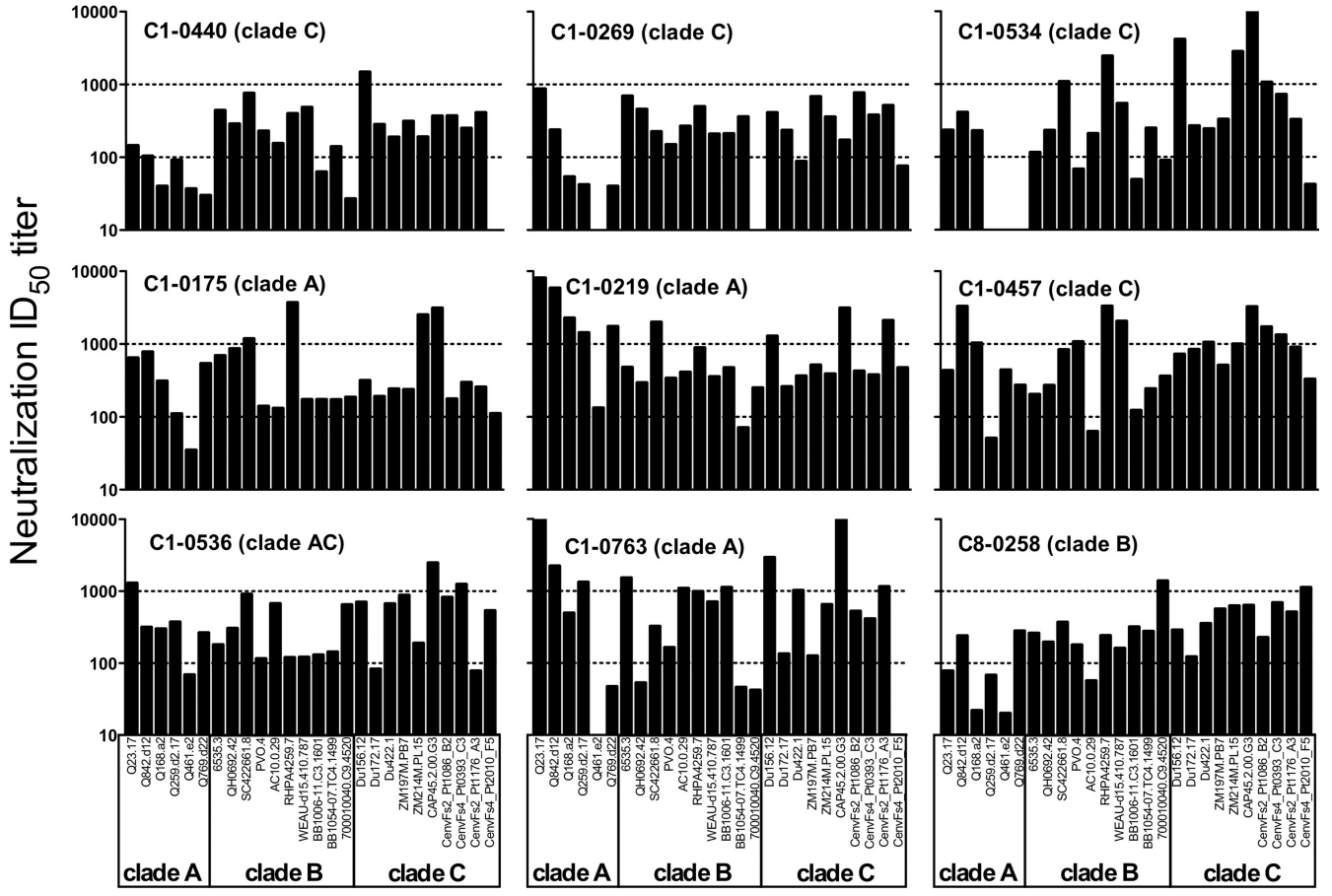

FIG. 2. ID_{50} titers of the 9 best neutralizers against a panel of 26 tier 2 viruses. Plasma neutralizing ID_{50} titers (reciprocal plasma dilutions) against 6 subtype A viruses, 10 subtype B viruses, and 10 subtype C viruses are shown.

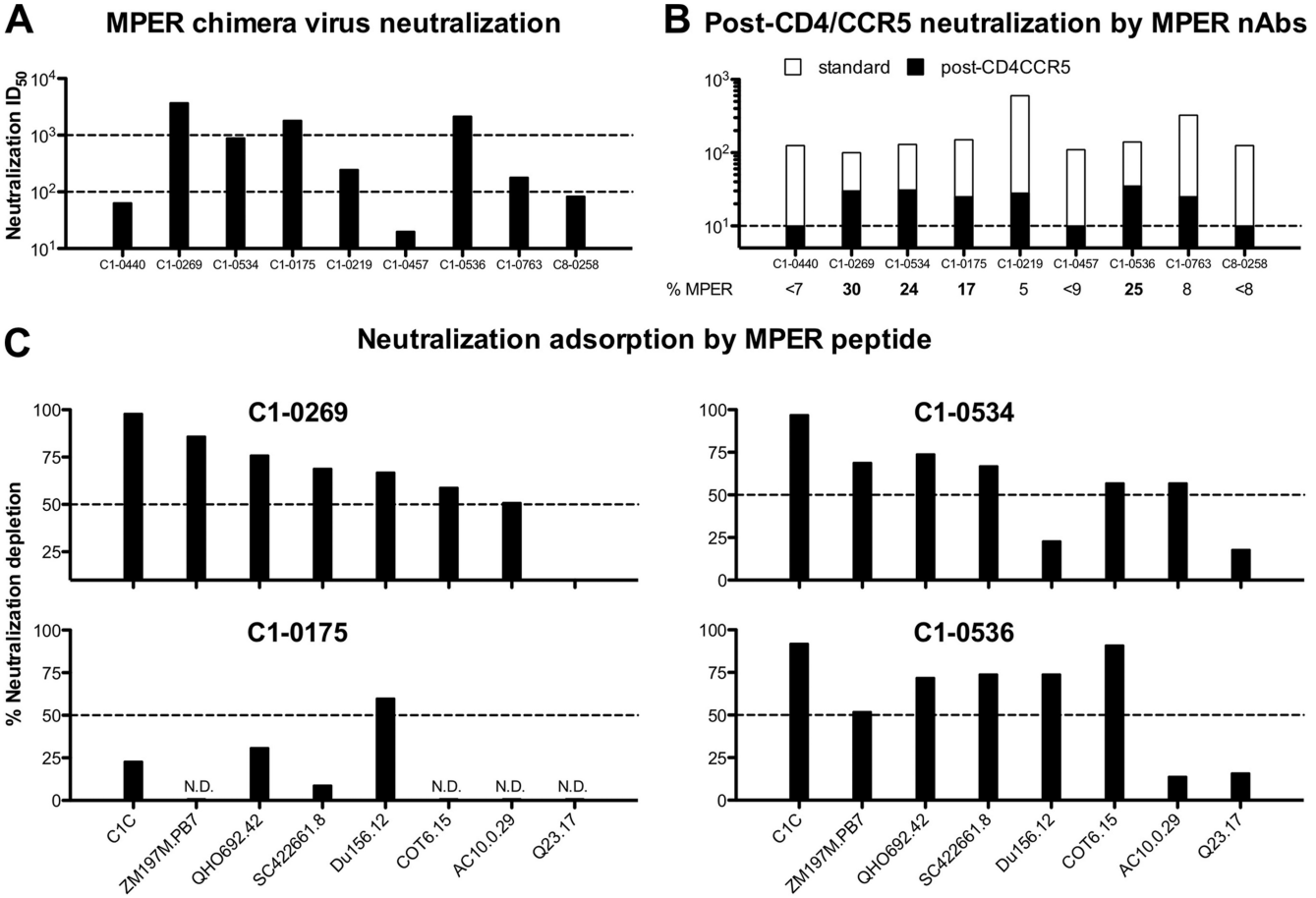

FIG. 3. Plasma neutralization directed to the gp41 MPER. (A) Neutralizing activities against an HIV-2 chimera containing a full HIV-1 clade B MPER graft are plotted as ID_{50} titers (reciprocal plasma dilutions). (B) Post-CD4/CCR5-engaged neutralization against a JR-FL SOS mutant mediated by MPER nAbs, shown as filled bars overlaid on open bars that represent neutralization (ID_{50} titers [reciprocal plasma dilutions]) against the same virus in the standard format (where the plasma and virus are incubated before receptor binding). Percentages of MPER-mediated neutralization, calculated as (ID_{50} in the post-CD4/CCR5 format)/(ID_{50} in the standard format), are given below each stacked bar. (C) Breadth of neutralization as probed by the effects of MPER peptide adsorptions on the neutralization of various pseudoviruses. Data are plotted as the reduction in the percentage of neutralization from that with the no-peptide treatment. In controls, the peptide adsorbed all MAb 2F5 and 4E10 neutralization (not shown). N.D., not detected.

proportionately lower responses to V2, C3, and C3/V4 (see Fig. S1D). Although insufficient material was obtained to examine the neutralization breadth panel shown in Fig. 2, neutralization of SF162 by purified IgG from a C1-0536 CVL specimen was detected at a 50% effective concentration (EC_{50}) of 10.5 μg/ml. This suggests that neutralizing IgG can exude into genital secretions, although it is not clear how the titer compares to that in plasma samples.

None of the plasma samples demonstrated any autoreactivity in the Athena Luminex assay (not shown). However, 7/9 subjects had anti-cardiolipin antibodies (not shown). This is consistent with previous findings that anti-cardiolipin antibodies correlate with neutralization breadth (41).

Cross-neutralizing activities targeting the gp41 MPER. We first investigated whether nAbs directed to the gp41 MPER were the basis for the broad neutralization seen in our 9 plasma samples. By use of a chimeric pseudovirus bearing an HIV-2 Env engrafted with a clade B HIV-1 MPER region (3, 37), four plasma samples (C1-0269, C1-0534, C1-0175, and C1-0536) exhibited nAb ID_{50} titers of approximately 1,000 or greater, and another two (C1-0219 and C1-0763) had ID_{50} titers of ~200 (Fig. 3A). All but one of these plasma samples also neutralized a similar chimera containing a consensus clade C MPER (not shown). An exception was plasma C1-0219, which had an ID_{50} of 81 against the clade C MPER chimera, approximately 3-fold lower than its titer against the clade B chimera.

The same six plasma samples that exhibited neutralizing activity against the MPER chimeras described above were also positive in the post-CD4/CCR5 assay (Fig. 3B, where MPER activity is shown as filled bars). This assay makes use of a JR-FL SOS mutant virus where fusion ensues only after a disulfide bond between gp120 and gp41 is broken by a reducing agent, allowing partitioning and analysis of neutralization activity that acts before or after receptor binding (1, 5, 21). MPER nAbs have the ability to mediate neutralization after the virus has engaged CD4 and CCR5 receptors. The proportion of the total neutralizing activity contributed by MPER

nAbs can be determined by comparing the post-CD4/CCR5 titers with those obtained in the standard format, where the SOS virus and nAbs are mixed before receptor binding (5, 21). The four plasma samples with the highest MPER activity in the chimera assays (C1-0269, C1-0534, C1-0175, and C1-0536) contained gp41 MPER activity that constituted 17 to 30% of the total neutralizing activity (Fig. 3B, % MPER). C1-0219 and C1-0763 had lower titers of MPER nAbs, indicating that these types of nAbs did not make a significant contribution to JR-FL neutralization in these two plasma samples.

To determine whether MPER nAbs were responsible for broad cross-neutralization, we performed depletion experiments on the four plasma samples that appear to have the highest proportion of MPER-neutralizing antibodies (Fig. 3B), using an MPER peptide immobilized onto magnetic beads (37). Depleted plasma samples were then tested for neutralization of heterologous tier 2 viruses. Control experiments showed that the peptide removed >95% of the neutralizing activity of the MPER MAb 4E10 (not shown). For plasma samples C1-0269, C1-0534, and C1-0536, MPER peptides removed >90% of the neutralizing activity against the HIV-2/HIV-1 MPER chimeric virus C1C (Fig. 3C). The MPER peptide also removed >50% of the neutralizing activity against most of a panel of 7 clade B and C reference viruses (Fig. 3C). In contrast, the MPER peptide did not remove activity from plasma C1-0175 against C1C and two heterologous isolates that were neutralized by the nonadsorbed sample, although MPER antibodies in this plasma sample were moderately responsible for Du156.12 neutralization. This result could reflect either a low level of background activity against the scaffold HIV-2 or a failure of the MPER peptide to fully mimic the MPER epitope found on the virus particle. Neutralization of the subtype A Q23.17 isolate was not significantly affected by the depletion of MPER antibodies in any of the three samples. Similarly, neutralization of Du156.12 by plasma C1-0534 and neutralization of AC10.0.29 by plasma C1-0536 appeared not to be mediated by MPER antibodies. All three viruses are sensitive to MAb 4E10, so these observations are likely not explained by any overt MPER resistance on the part of these particular viruses. This suggests that these viruses are also sensitive to another neutralizing specificity present in these plasma samples.

Specificities of MPER-directed plasma neutralization. To investigate the precise residues within the MPER recognized by these plasma samples, we made use of chimeric viruses where fragments of the HIV-1 MPER were engrafted into HIV-2 7312A. Plasma samples C1-0269, C1-0534, and C1-0536 showed high neutralization titers against the C4, C4GW, and C8 chimeras, which contain substitutions in the C-terminal portions of the MPER, suggesting 4E10/Z13-like nAbs (Fig. 4A). All three plasma samples also showed low-level neutralization of C6, which is the chimera neutralized only by MAb 4E10. To further pinpoint these specificities, we used a series of MPER alanine mutant pseudoviruses in the clade C COT6.15 Env background—a virus against which the MPER peptide adsorbed most of the plasma neutralizing activity (Fig. 3C). This revealed a focus on residues F673 and D674 in the C-terminal part of the MPER for all three plasma samples, further suggesting 4E10/Z13-like epitopes (Fig. 4B). Plasma sample C1-0269 was also sensitive to the W672A mutation, which ablates MAb 4E10 neutralization (37). Plasma sample C1-0536 and, to a lesser extent, C1-0269 were partially dependent on W670 (Fig. 4B). The fact that the latter residue is present in grafts C4GW and C8 but not in C4 might explain the difference in the neutralization activity against these two chimeras by plasma C1-0536. Plasma samples C1-0269 and C1-0536 were also sensitive to other C-terminal positions in the MPER, notably L679A and W680A, the latter of which also affects 4E10 neutralization (37). The binding of purified plasma IgG from C1-0269 and C1-0534 to the MPER epitope, as measured by surface plasmon resonance (expressed as the dissociation constant [K_d]), was 112 nM and 268 nM, respectively. These values are approximately 3.6- and 8.7-fold lower than the K_d of the binding of MAb 4E10 to the MPER epitope (100). No antibodies binding to the 2F5 epitope were detected, indicating that the MPER responses in these plasma samples were directed predominately to the 4E10 epitope.

We also examined neutralization using the JR-FL SOS mutant virus in the post-CD4/CCR5 assay format. In this assay, substitution of residues known to affect 2F5 (D664A) and 4E10 (W672A) ablated the neutralizing activities of 2F5 and 4E10, respectively. As with the COT6.15 MPER mutants, there was a focus on residues W672, F673, and D674 for neutralization in all three plasma samples (Fig. 4C). The role of D674 was less clear in this case, because the JRFL D674A mutant virus is less sensitive to both 2F5 and 4E10 (not shown). This was not the case for 4E10 neutralization of the COT6.15 D674A mutant (37). Given that this epitope resides well outside the known epitope for 2F5, we can infer that D674 mutations can result in a globally resistant phenotype, characterized by a greater overall resistance to distal nAb specificities. This contrasts sharply with the "globally sensitive" nature of many of the MPER mutants (as evidenced by plasma ID_{50} titers below a 1:1 ratio in Fig. 4B and C). Thus, the D674-dependent activity should be viewed with caution, considering its ability to globally regulate neutralization sensitivity. Nevertheless, the present data indicate that the C-terminal portion of the MPER, in particular W672, F673, and possibly D674, contributes to the broad neutralization activities of these plasma samples, consistent with 4E10/Z13-like activity.

Cross-neutralizing activities targeting gp120 and the CD4bs. gp120 monomer adsorption provides a way to partition gp120-reactive nAbs from nAbs that recognize either quaternary epitopes or epitopes in gp41 (26, 57, 58, 89). ConC gp120-depleted plasma was tested for neutralizing activity. In each case, for the eight subjects tested, binding to the matched ConC gp120 by ELISA was eliminated, confirming the efficacy of adsorptions (not shown). Plasma sample C8-0258 was not included in this analysis, because its ID_{50} against the ConC virus was too low for any effects of depletion to be reliable. Furthermore, another study describing plasma C8-0258 reported that gp120 depletion did not affect neutralization against the JR-FL isolate (76).

Using >50% depletion as the cutoff for significance, plasma neutralization of the matched ConC pseudovirus was depleted by ConC gp120 adsorption in all cases (Table 2). For four plasma samples—C1-0175, C1-0219, C1-0457, and C1-0763—neutralization of one or more other isolates was also depleted, suggesting the presence of broad nAbs directed to the gp120 monomer. In contrast, the neutralizing activities of the remain-

A

Virus	C1-0269	C1-0534	C1-0536	MPER sequence	2F5	4E10	Z13e1
7312A	<40	<40	85	NMYELQKLNSWDVFGNWFDLASWVKYIQYGVYIV	>10	>10	>10
C1C	2,289	1,724	2,973	NMYELLALDSWKNLWNWFDITKWLWYIKYGVYIV	>10	0.048	0.282
C3	<40	<40	131	NMYELLALDKWASLWNWFDLASWVKYIQYGVYIV	0.075	>10	>10
C7	<40	<40	75	NMYELQALDKWAVFGNWFDLASWVKYIQYGVYIV	0.031	>10	>10
C6	254	264	156	NMYELQKLNSWDVFGNWFDITSWIKYIQYGVYIV	>10	0.077	>10
C4	1,735	1,313	574	NMYELQKLNSWDVFGNWFDITKWLWYIKYGVYIV	>10	0.198	1.132
C4GW	2,280	1,362	4,122	NMYELQKLNSWDVFWNWFDITKWLWYIKYGVYIV	>10	0.126	0.374
C8	2,968	1,785	2,325	NMYELQKLNSWDSLWNWFDITKWLWYIKYGVYIV	>10	0.145	0.786

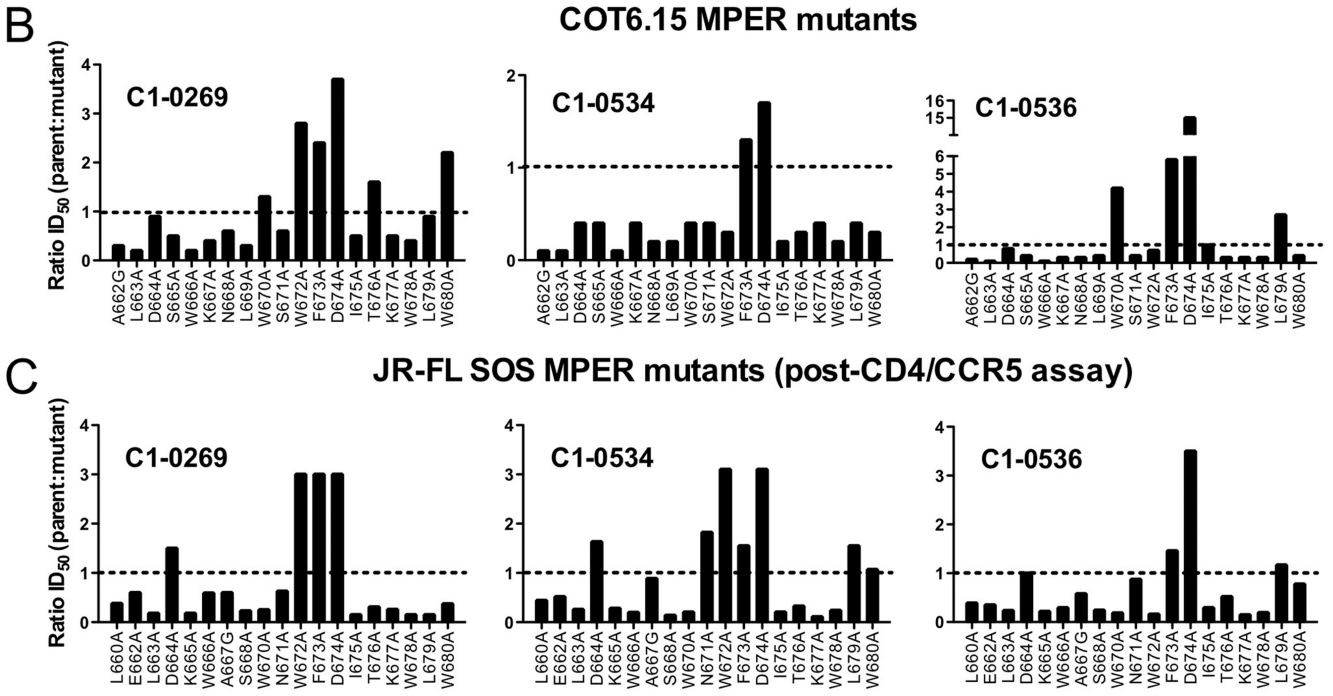

FIG. 4. Fine specificity of MPER-neutralizing activities. Three plasma samples exhibiting significant MPER breadth were further analyzed to determine the precise MPER epitopes. (A) Plasma samples were first assessed for neutralization of HIV-2 chimeras engrafted with various segments of the HIV-1 MPER. The ID_{50} titers (reciprocal plasma dilutions) are given for plasma samples C1-0269, C1-0534, and C1-0536. The concentrations (μg/ml) equivalent to the IC_{50} are shown for the monoclonal antibodies (2F5, E10, Z13e1). (B) The activities against a series of MPER mutants of the COT6.15 virus were assessed. The ratio of the neutralization ID_{50} against the parent virus to that against the mutant virus is shown. (C) Neutralizing activities against a series of MPER mutants in the JR-FL SOS background were assessed using the post-CD4/CCR5 format (focused only on MPER activity). The ratio of the neutralization IC_{50} against the parent virus to that against the mutant virus is shown.

ing four plasma samples against other isolates were not effectively depleted by gp120. These data suggest that anti-gp120 nAbs were partially responsible for neutralization breadth in approximately 50% of the plasma samples tested, including all the level 3 neutralizers.

To better understand the nature of the cross-neutralizing gp120-specific nAbs, depletions were performed using a ConC core gp120 lacking the V1V2 and V3 variable loops. In the case of C1-0457, core gp120 was as efficient as gp120 at adsorbing the ConC-neutralizing activity (Table 2). However, it was less effective at depleting the neutralizing activity of C1-0219 and almost completely ineffective at depleting neutralization in plasma samples C1-0763 and C1-0175. This suggests that the neutralizing activity of plasma C1-0457 targets an epitope in the core of gp120, while the plasma from the other three subjects depends on the variable loops to various extents.

We also investigated the ability of gp120 with point mutants of key residues in the CD4bs (D368R) and CD4i (I420R) epitopes to deplete neutralizing activity (57). The gp120 CD4bs (D368R) mutant was less efficient than wild-type gp120 at adsorbing the neutralizing activity of plasma C1-0219 against several isolates, indicating that CD4bs nAbs were responsible for some of the neutralization breadth in this plasma sample (Table 2). However, the gp120 I420R mutant did not appreciably affect gp120 adsorption of plasma C1-0219, and neither of the mutant gp120s affected the adsorption of the other plasma samples.

To further investigate the CD4bs nAbs in plasma sample C1-0219, we examined the ability of a resurfaced gp120 core protein (RSC3) to deplete neutralizing activity. RSC3 consists of an SIV gp120 core engrafted with key residues important for the binding of CD4bs antibodies (114), making it a highly specific probe for binding only CD4bs nAbs. The RSC3 protein depleted more than 40% of the neutralizing activity of plasma

TABLE 2. Adsorption of neutralizing antibodies using recombinant envelope proteins[a]

Plasma sample	Virus	ID_{50} with blank beads	gp120		gp120 core		gp120 D368R		gp120 I420R	
			ID_{50}	% Ads	ID_{50}	% Ads	ID_{50}	% Ads	ID_{50}	% Ads
C1-0440	ConC	563	143	**75**			206	11	212	12
	CAP45.G3	172	148	14						
	Du156.12	357	294	18						
C1-0269	ConC	3,152	1,039	**67**			1,202	5	847	0
	CAP45.G3	734	923	0						
	Du156.12	341	307	10						
C1-0534	ConC	629	268	**57**			307	6	329	10
	CAP45.G3	14,776	8,740	41						
	Du156.12	6,951	6,022	13						
C1-0175	ConC	1,578	417	**74**	1,265	**20**	266	0	367	0
	CAP45.G3	3,436	<60	>**98**			<60	0	<60	0
	Du156.12	320	239	25						
C1-0219	ConC	2,711	880	**68**	1,652	**39**	1,508	**23**	1,253	14
	CAP45.G3	3,713	988	**73**			1,939	**26**	1,772	**21**
	Du156.12	1,013	480	**53**			931	**45**	502	2
	SC422661	338	<60	>**82**			263	**60**	124	19
C1-0457	ConC	2,428	<60	>**95**	159	**93**	324	11	<60	0
	CAP45.G3	4,006	<60	>**99**			<60	0	<60	0
	Du156.12	1,129	154	**86**			242	8	120	−3
	SC422661	145	<60	>**59**			<60	0	<60	0
C1-0536	ConC	489	131	**73**			120	0	187	11
	CAP45.G3	2,126	3,554	0						
	Du156.12	913	764	16						
C1-0763	ConC	22,392	5,608	**75**	19,699	12	6,606	4	5,671	0
	CAP45.G3	13,037	1,523	**88**			2,467	7	2,834	10
	Du156.12	1,578	487	**69**			612	8	713	14

[a] Plasma fractionation was carried out with monomeric gp120 and gp120 mutants. Plasma samples were adsorbed with wild-type ConC gp120-coated beads, and the neutralizing ID_{50}s (reciprocal plasma dilution) against various pseudoviruses were determined. Blank beads were used as a control. The percentage of neutralization (% Ads) directed to gp120 was calculated as the reduction in the ID_{50} by adsorption with wild-type gp120 from the ID_{50} with blank beads [(ID_{50} with blank beads − ID_{50} with wild-type gp120)/(ID_{50} with blank beads)]. A similar procedure was used to evaluate the adsorptive abilities of a gp120 core lacking the V1V2 and V3 loops, a gp120 variant containing a mutation in the CD4 binding site (D368R), and a gp120 variant containing a mutation affecting the detection of CD4i epitope-directed antibodies (I420R). For these, CHAVI plasma sample C8-0258 was not tested, because its ID_{50} titer against the ConC virus was too low. The percentage of neutralization directed to each mutant was calculated as [(ID_{50} with the gp120 variant − ID_{50} with wild-type gp120)/(ID_{50} with blank beads)]. Values equal to or greater than 50% for wild-type gp120, and values equal to or greater than 20% for the mutants, are shown in boldface. In cases where no neutralization was observed at the lowest dilution (1:60), a value of <60 is given.

C1-0219 against six viruses (Fig. 5A), consistent with the gp120 fractionation data (Table 2). In control experiments, RSC3 completely adsorbed the neutralization activity of VRC01 against six of seven primary viruses. A P363N mutant of RS3C that affects the CD4 binding loop was unable to block VRC01 or plasma C1-0219 neutralization (114), confirming the specificity of the interference (Fig. 5A).

We next examined the binding of plasma C1-0219 to native Env trimers in BN-PAGE shift assays and the effects of mutations known to ablate the binding of CD4bs MAbs b12 and VRC03. Plasma C1-0219 bound effectively to trimers, as did CD4bs MAbs b12 and VRC03 (Fig. 5B) (7). D368R mutant trimers completely knocked out the binding of both CD4bs MAbs b12 and VRC03 and partially reduced the binding of plasma C1-0219. This is consistent with the effect of the D368R mutation on gp120 depletion (Table 2). No D368R-sensitive binding activity was detected in any of the other plasma samples by BN-PAGE shifts (not shown). To further characterize the specificity of plasma C1-0219, we examined the effects of a mutation at residue W479, a known CD4 contact residue. This mutant completely ablated VRC03 binding but had no effect on b12, revealing a difference in the fine specificities of these two MAbs. Plasma C1-0219 was unaffected by the W479G mutant, suggesting that its CD4bs nAbs are more akin to b12 than to VRC03 (Fig. 5B).

Broadly neutralizing antibodies targeting HIV-1 gp120 variable loops. We next investigated variable-loop-dependent neutralizing activity by using panels of chimeric viruses (73), making use of virus pairs that exhibit markedly different sensitivities to neutralization by an individual plasma sample. Seven of the nine plasma samples neutralized one of the parental pairs and were therefore tested against their chimeras in which the variable loops (V1V2, C3-V4, V4, or V5) of the sensitive virus were engrafted into the resistant Env background. Four plasma samples (C1-0440, C1-0219, C1-0536, and C8-0258) showed no preferential activity against any of the chimeras and thus were not investigated further. Plasma samples C1-0175 and C1-0534 neutralized three or all four of the chimeras, respectively, suggesting that they target multiple or complex epitopes (not shown). In contrast, plasma sample C1-

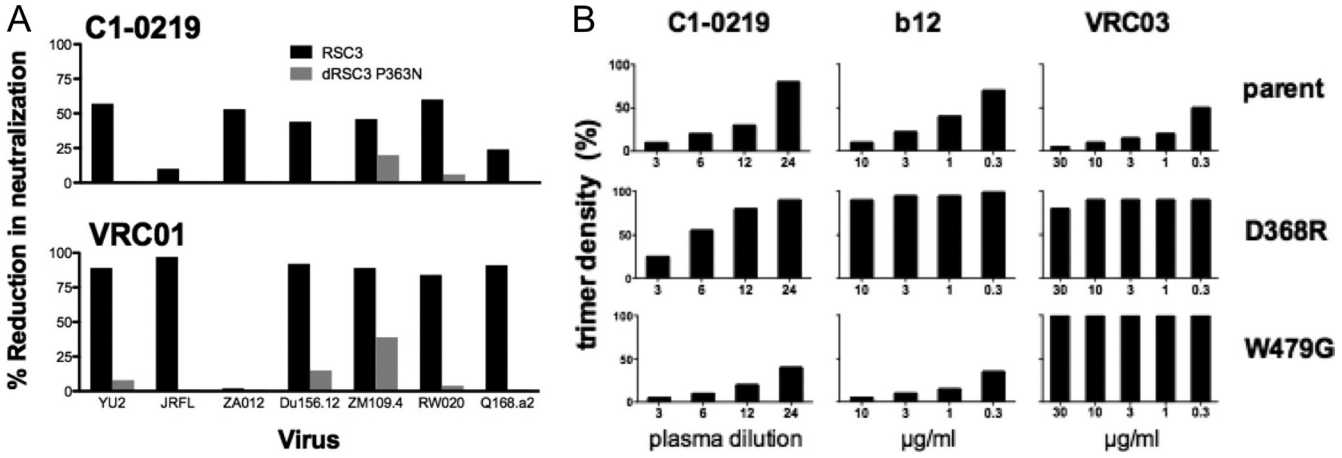

FIG. 5. Detection of CD4bs nAbs. (A) Effects of interference by resurfaced cores RSC3 and dRSC3 P363N on the neutralization of various viruses by plasma C1-0219 or MAb VRC01. (B) Binding of plasma C1-0219 and MAbs b12 and VRC03 to native JR-FL SOS Env trimers as determined by BN-PAGE and the effects of mutations D368R and W479G, which affect the CD4bs. Trimer binding is assayed by a loss in trimer staining density (shown as bars), since trimers are complexed with nAbs.

0763 neutralized only the V1V2 loop-engrafted chimeras made between the sensitive CAP45 and the resistant CAP84 virus (Fig. 6A). This result was replicated with a second virus pair (not shown), confirming that plasma C1-0763 recognized a V1V2 loop-dependent epitope.

To determine whether the anti-V1V2 nAbs in C1-0763 cor-

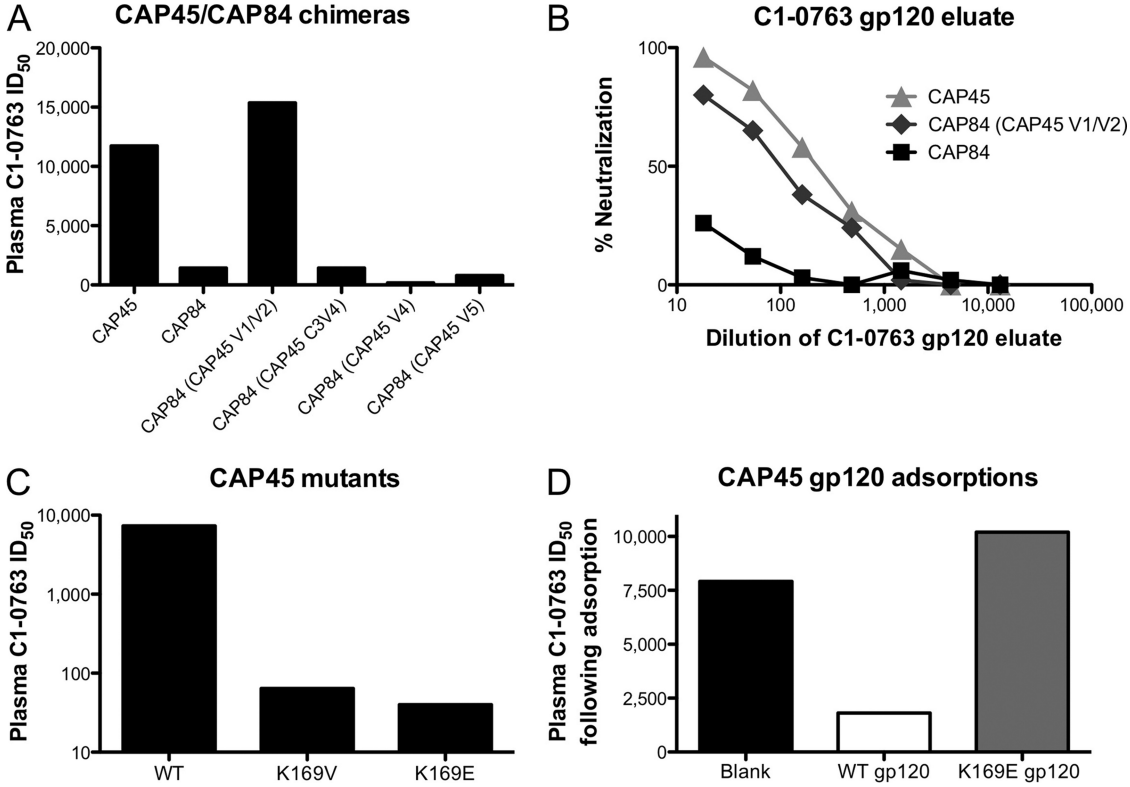

FIG. 6. Detection of V1V2 nAb activity in plasma sample C1-0763. (A) Neutralization by plasma C1-0763 (ID_{50} titer [reciprocal plasma dilution]) of the CAP45 virus and the CAP45 V1V2 engrafted into the background of the less-sensitive virus CAP84. (B) The eluate from plasma C1-0763 adsorption on gp120 beads mediates V1V2-directed neutralization, as indicated by neutralization of the chimeric CAP84 virus containing the CAP45 V1V2 loop. Parental viruses CAP45 and CAP84 were used as positive and negative controls, respectively. (C) Effects of mutations at position K169 in the V2 loop of the CAP45 virus on neutralization by plasma C1-0763. Each bar represents the ID_{50} titer (reciprocal plasma dilution) obtained with a wild-type (WT) or mutant CAP45 virus. (D) Effect of K169E mutation on the ability of monomeric CAP45 gp120 to adsorb plasma neutralization against the CAP45 virus. Each bar represents the ID_{50} titer obtained with the plasma samples after adsorption with blank beads, wild-type gp120, or gp120 K169E.

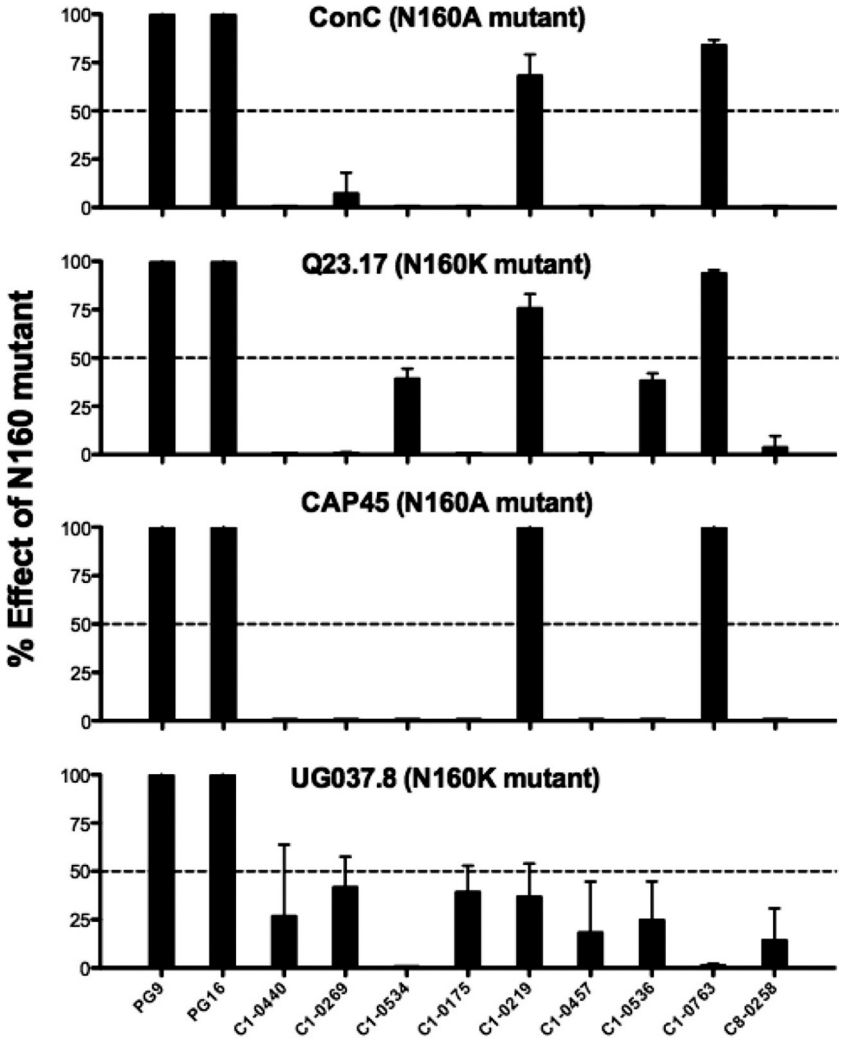

FIG. 7. Mapping of neutralizing activity sensitive to the replacement of residue N160. The neutralization activities of each of our plasma samples and MAbs PG9 and PG16 were assayed against four viruses (ConC, Q23.17, CAP45, and UG037.8) bearing mutations at residue N160 in the gp120 V2 loop. Data are expressed as the percentage of the reduction in titer relative to the neutralization of the unmutated parent virus.

responded to those that were depleted by the gp120 monomer, we assayed the antibodies eluted from the gp120-coated beads against the sensitive and resistant virus pair as well as the V1V2 chimera. We found that the CAP45 virus and the chimera containing the CAP45 V1V2 region were both sensitive to the gp120 eluate, while the CAP84 virus was resistant (Fig. 6B). Furthermore, using V2 loop point mutants, we identified residue K169 as crucial for neutralization, with both 169V and 169E having dramatic effects (Fig. 6C). Adsorption experiments using a CAP45 gp120 variant with a K169E mutation completely ablated the depleting abilities of gp120 for this plasma (Fig. 6D). This indicates that the V2-directed gp120-adsorbable neutralizing antibodies in plasma C1-0763 depend on residue K169 in the V2 loop. Thus, while the antibodies in plasma C1-0763 targeted a region similar to that targeted by MAbs PG9 and PG16, they were unlike PG9 and PG16, whose V2 activity depends on a quaternary structure and is therefore not absorbable by gp120.

Detection of PG9/PG16-like neutralizing activity. The glycan at position 160 in the V2 loop is a critical part of the epitope of MAbs PG9 and PG16, and viruses with a mutation at this site are often used to define this specificity (although differences between isolates have been noted) (112). To investigate this, we examined N160-dependent activity in four different viral Env backgrounds. As expected, MAbs PG9 and PG16 were unable to neutralize the N160 mutants of any of the ConC, Q23.17, CAP45, and UG037.8 Envs (Fig. 7). Using this approach, we found that the neutralizing activity in two of the nine plasma samples was sensitive to a mutation at this position. The neutralization titers of plasma samples C1-0219 and C1-0763 were substantially reduced by N160A mutations in the ConC and CAP45 viruses and by an N160K mutation in the Q23.17 virus (Fig. 7). None of the other plasma samples showed a significant effect (Fig. 7). The UG037.8 virus N160K mutant did not reveal activity in any of the plasma samples, underscoring the various results seen with different isolates.

A **Effect of V2 single point mutants in CAP45.G3**

Mutant	Region	Fold decrease in ID_{50}		Fold increase in IC_{50}	
		C1-0219	C1-0763	PG9	PG16
CAP45 N160A	V2	23	>300	>1000	>1000
CAP45 L165A		1.0	9.1	2.4	2.0
CAP45 R166A		0.7	3.6	1.0	1.3
CAP45 D167N		1.0	0.7	3.1	3.5
CAP45 K169E		38	>300	>1000	>1000
CAP45 K171E		10	2.3	8.7	21

2-5 fold
5-10 fold
10-100 fold
>100 fold

B **Effect of V1V2 and V3 single point mutants ConC**

Mutant	Region	Fold decrease in ID_{50}		Fold increase in IC_{50}	
		C1-0219	C1-0763	PG9	PG16
ConC V127A	V1V2	4.6	2.9	27	57
ConC R132A		0.7	3.5	0.5	0.6
ConC S158A		0.8	0.6	0.9	0.9
ConC F159A		4.5	3.3	11	266
ConC N160A		5.4	9.3	>1000	>1000
ConC L165A		0.9	1.0	2.0	2.0
ConC R166A		0.9	3.7	0.5	0.7
ConC D167N		1.1	0.3	0.6	1.1
ConC K168A		2.1	10	0.1	0.1
ConC K169E		2.0	2.9	>1000	>1000
ConC K171A		3.4	4.3	5.9	23
ConC I181A		5.1	3.0	13	17
ConC K305A	V3	2.7	2.5	2.0	1.6
ConC I307A		0.7	0.6	4.3	5.2
ConC R308A		0.8	1.7	0.5	0.8
ConC I309A		2.0	2.2	3.1	2.2
ConC Q315R		1.1	0.9	0.9	0.9
ConC F317A		1.6	2.3	2.0	1.5
ConC Y318A		2.2	1.6	1.5	1.2
ConC N332A		0.4	0.5	0.3	0.4

FIG. 8. Effects of mutations in V1V2 and V3 on neutralization by plasma samples C1-0219 and C1-0763. The neutralizing activities of plasma samples C1-0219 and C1-0763 and of MAbs PG9 and PG16 were tested against single point mutants with mutations in the V2 region of CAP45.G3 (A) and the V1V2 and V3 regions of ConC (B). Data are expressed as the ratio of the ID_{50} against the parent virus to that against the mutant virus. Mutations that resulted in a significant fold change are highlighted in colors shading from yellow (2- to 5-fold) to red (>100-fold). Amino acid numbering is based on the sequence of HXB2.

To further determine whether activities in plasma samples C1-0219 and C1-0763 shared residues with PG9 and PG16, we used a series of V2 and V3 point mutants in two different Env backgrounds. N160A and K169E mutations in both Env backgrounds had profound effects on PG9 and PG16, which were also affected, albeit to a lesser degree, by V127A, F159A, K171A, K171E, and I181A, depending on the background Env (Fig. 8). N160A and K169E had similar profound effects on both plasma samples in the CAP45 background, where C1-0219 was also affected by K171E, while C1-0763 was also affected by L165A.

In the ConC background, both plasma samples were affected by the N160A mutant, but very little effect was seen with the K169E mutant. In addition, mutations at residues 159 and 181 reduced the neutralization titers of plasma samples C1-0219 and C1-0763 at least 3-fold (Fig. 8). Plasma C1-0219 neutralization was also reduced by a V127 mutant in the V1 loop, while that of plasma C1-0763 was also reduced by an R132A mutation in the V1 loop and by R166A and K168A mutants in the V2 loop. The latter two sites distinguished C1-0763 from PG9 and PG16.

In the JR-FL background, the R166A mutant knocked down plasma C1-0219 (see Fig. S3 in the supplemental material), indicating that the precise substitutions affecting C1-0219 neutralization in each Env background differed. Collectively, these findings suggest that plasma C1-0219 may contain a swarm of V2 nAbs that recognize slightly different epitopes on the V2 loop, depending on the Env isolate. Alternatively, these knockdown effects could be steric and could actually affect binding to a neighboring site. In addition, the presence of CD4bs neutralization activity in this plasma sample may significantly inhibit attempts to accurately determine V2 knockout mutants, whose effects may be camouflaged by the retention of background CD4bs activity.

In contrast to the knockdown effects on neutralization of ConC by plasma C1-0763 (see Fig. S2 in the supplemental material), this plasma sample was not affected by V2 mutations in the JR-FL background (see Fig. S3 in the supplemental material). These differential effects could be explained by a failure of the V2 activity to cross-react with JR-FL Env and/or the presence of additional nAb activities in this plasma sample that mask the effect of the V2 antibodies against the JR-FL virus. The neutralization titers of plasma C1-0763 were significantly lower against JR-FL (ID_{50}, 325) than against ConC (ID_{50}, >10,000), suggesting that the potent V2 loop-directed activity may simply not cross-react with the JR-FL isolate.

The use of mutants in the JR-FL background also revealed activity in plasma C1-0457 that was not detected against either ConC or CAP45. This affected residues 165, 166, and 167 in the V2 loop. One possibility is that this neutralizing activity is restricted in breadth, such that it does not cross-react with ConC or CAP45. Alternatively (or additionally), the effects of the V2 activity may be masked by other neutralizing specificities effective against the ConC Env but not against JR-FL. In support of the latter possibility, plasma C1-0457 neutralized the ConC virus with an approximately 10-fold higher titer.

We also examined V3 mutations (see Fig. S3 in the supplemental material), because these are also components of the PG9/PG16 epitope. Neutralization by plasma C1-0219 was affected by the R304A mutation in the N-terminal base of the V3 loop in JR-FL and by the K305A, I309A, and Y318A mutations in ConC. We found no evidence for competition of JR-FL neutralization using V3 peptides (experiments were performed as described previously [7]), suggesting that this V3 reactivity does not depend on linear V3 sequences. Plasma C1-0763 was sensitive to mutations in and around the C-terminal portion of the V3 loop, including E320A, G323C, and D324A in JR-FL and K305A, I309A, and F317A in ConC. Given that no evidence was found for V2 knockouts in the JR-FL background, it is possible that this V3 dependency is a distinct neutralizing specificity and is not linked to V2. Overall, the effects in V3 were minor compared to those in V2, with some overlap with the sites targeted by MAbs PG9 and PG16.

Cross-neutralizing antibodies targeting glycans. Glycans on the gp120 outer domain are also the target of nAbs, as typified by MAb 2G12. Thus, we explored mutants designed to knock out gp120 glycans. N332A and N339A mutations of JR-FL knocked down plasma samples C1-0763 and C1-0175, respectively (see Fig. S3 in the supplemental material). In the ConC background, it was difficult to determine the effect of the N332A mutation, because in this Env background the mutant was globally sensitive (not shown). We therefore investigated the effects of N332A mutants in four other virus Envs (Fig. 9A). This revealed that N332A knocked out C1-0763 neutralization of the clade B isolates JR-FL and TRO.11, but not the clade A (Q23.17) or C (Du156.12) viruses. The TRO.11 N332A mutant also became more resistant to neutralization by plasma samples C1-0440 and C1-0457. Plasma C1-0440 was also sensitive to the N332A mutant in the Q23.13 background. This suggested that at least three of the nine plasma samples contained broadly neutralizing antibodies that relied on the N332 residue.

Residue N332 is a central contact of the MAb 2G12 epitope (14, 87, 94). We therefore tested for 2G12 competition for binding to JR-FL gp120 by ELISA. Plasma C1-0763 was able to partially block the binding of biotinylated 2G12, whereas plasma C1-0219 had no effect (Fig. 9B). As expected, unlabeled 2G12 was able to compete biotinylated 2G12 effectively. This suggests that the N332A-sensitive activity in plasma C1-0763 overlapped the 2G12 epitope. It is unlikely to be exactly similar to 2G12, because it was not dependent on N295, which is a major core contact for MAb 2G12, along with N339, N386, and N392 (87).

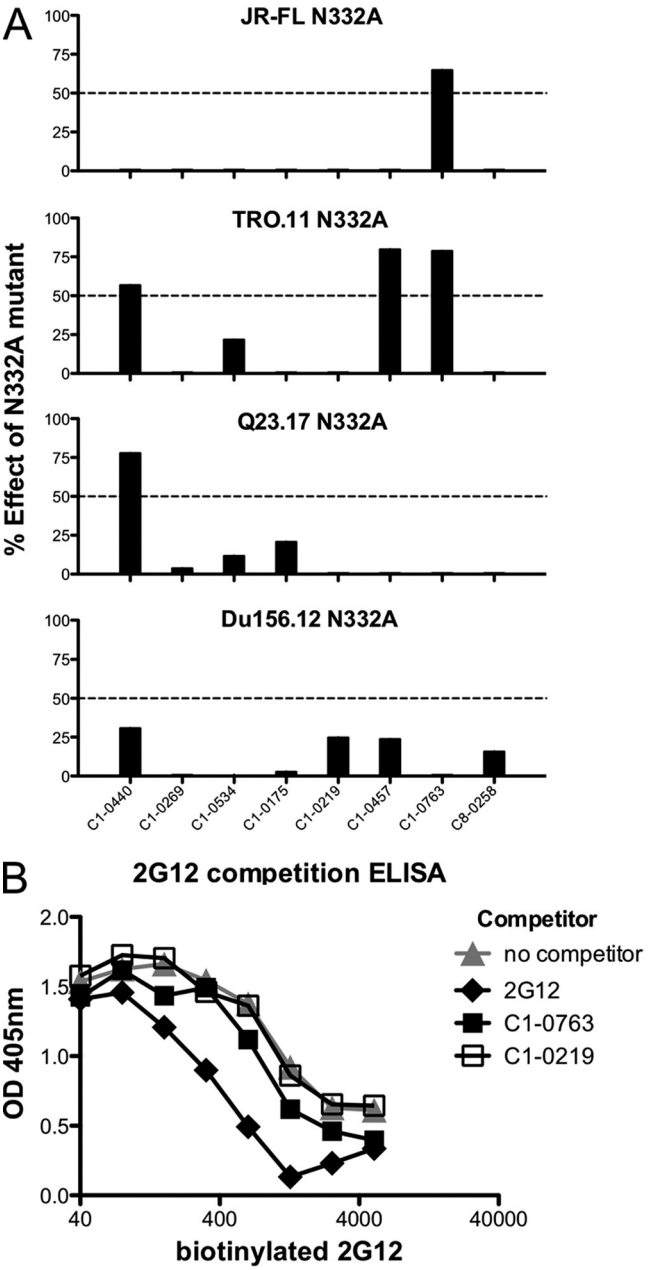

FIG. 9. Sensitivity of plasma neutralization to N332A mutation and overlap with the MAb 2G12 epitope. (A) The effects of N332A mutation in the contexts of various viruses on plasma neutralization were determined. (B) Abilities of plasma samples to compete with MAb 2G12 for gp120 binding. Plasma samples at a fixed dilution of 1:50 or 2G12 at a fixed concentration of 10 μg/ml was used to compete against biotinylated 2G12, which was titrated against the plasma or 2G12-blocked immobilized JR-FL gp120.

DISCUSSION

A key question regarding broad plasma neutralization of HIV-1 is whether it is typically mediated by one (or a very few) broad and potent antibody or by the sum of the activities of a large number of narrowly focused neutralizing specificities. Studies exemplifying both of these scenarios have been reported (4, 95, 112). The present study represents an extensive collaborative effort of multiple laboratories employing a variety of mapping tools and provides evidence that two or more distinct antibody specificities can sometimes mediate breadth in a single subject (summarized in Fig. 10). Interestingly, bnAbs with the greatest magnitude and breadth targeted gp120, while those with less breadth recognized the MPER, and these two general specificities were largely mutually exclusive. In at least one case (C1-0219), two distinct bnAb specificities against gp120 were identified, one resembling PG9/PG16 and the other directed against the CD4bs. The fact that both specificities have been isolated as broadly neutralizing

Patient	MPER				CD4bs and CD4i			gp120 Core	V2 Variable Loop		PG9/PG16-like			2G12-like
	MPER Chimeras Fig 3A, Fig 4A	Post-CD4/CC5 Fig 3B, 4C	MPER Peptide Adsorp. Fig 3C	Epitope Mapping MPER Mutants Fig 4B,C	CD4bs, CD4i Protein Adsorp. Table 2	CD4bs RSC Interference Fig 5A	CD4bs mut SOS Env trimer Fig 5B	gp120 Core Adsorp. Table 2	V1/V2[b] Chimeras Fig 6A,B,C	gp120 Adsorp. with V2 mut. Fig 6 D	N160K Mutant Viruses Fig 7	V1V2 Mutant Viruses Fig 8A, B, SOM Fig 2,3	V3 Mutant Virus Fig 8B, SOM Fig 3	N332/339 Mutant Viruses Fig 9A, SOM Fig 3
C1-0440	-	-	*nd*	*nd*	-	-	-	-	-	-	-	-[d]	-[d]	+ N332
C1-0269	++ 4E10/Z13	++ 4E10/Z13	++	4E10/Z13	-	-	-	-	*nd*[a]	-	-	-[d]	-	-
C1-0534	++ 4E10/Z13	++ 4E10/Z13	++	4E10/Z13	-	-	-	-	-[c]	-	-	-[d]	-[d]	-
C1-0175	++	++	-/+	*nd*	-	-	-	+	-[c]	-	-	-[d]	-	+ N339
C1-0219	+	+/-	*nd*	*nd*	++	+	+	+	-	-	++	++ quaternary	+	-
C1-0457	-	-	*nd*	*nd*	-	-	-	++	*nd*[a]	-	-	-[d]	-	+ N332
C1-0536	++ 4E10/Z13	++ 4E10/Z13	++	4E10/Z13	-	-	-	-	-	-	-	-[d]	-[d]	-
C1-0763	+	+/-	*nd*	*nd*	-	-	-	+	++	++	++	++ monomeric	+	+ N332
C8-0258	-	-	*nd*	*nd*	-	-	-	*nd*	-	*nd*	-	-[d]	-[d]	-

[a] Plasma did not neutralize parental virus and could not be tested. [b] V1V2, C3-C4, V4 and V5 chimeras were tested. Data is reported for V1V2. [c] Neutralized ≥3 chimeras. [d] Mutations in V2 were found to affect neutralization (see SOM Figs 2,3), but none were PG9/PG16-like. *nd* = not done.

FIG. 10. Summary of broadly neutralizing antibody targets. The neutralizing epitopes targeted by the plasma antibodies from the nine subjects studied here can be described in six broad categories: MPER, CD4bs and CD4i, gp120 core, V2 variable loop, PG9/PG16-like, and 2G12-like. The methods utilized to define the specificities of neutralizing antibodies are indicated (along with the relevant figure and table numbers). Different colors in the shaded boxes indicate the presence of neutralizing antibody specificities circulating in each subject's plasma.

MAbs from this subject (10; J. R. Mascola, unpublished data) validates the presence of multiple plasma antibody specificities in a single individual, as well as the utility of mapping efforts to identify interesting epitopes for further study, including the isolation of novel broadly neutralizing MAbs. The presence of multiple broadly neutralizing antibodies circulating in plasma suggests that vaccines could, in theory, be designed to stimulate the immune response to productively develop multiple neutralizing antibody specificities that confer breadth.

We made use of a number of different methods to map the antibody specificities mediating broad neutralization in plasma from 9 chronically infected, antiretroviral-naïve subjects selected from a cohort of 308 patients infected mostly with subtypes A and C. The results of three different assays confirmed the presence of anti-CD4bs antibodies in plasma C1-0219. This included the use of ConC recombinant gp120 with a mutation at position 368 in the CD4bs that failed to deplete neutralizing activity, as well as the use of a YU2 resurfaced core protein that specifically binds CD4bs antibodies. A third method measured blue native gel band shifts and showed that C1-0219 efficiently shifted a JR-FL trimeric Env; this shift was lost when a protein containing the position 368 mutation was used, as with the prototype CD4bs MAbs, b12 and VRC03. None of the other 8 samples contained anti-CD4bs antibodies as determined by these methods, suggesting that broadly neutralizing CD4bs antibodies are induced relatively infrequently.

The presence of anti-MPER Abs was confirmed in at least 3 of the plasma samples in multiple assays. An HIV-2/HIV-1 MPER chimera detected high activity in 6 plasma samples, 3 of which were shown to mediate cross-neutralization via this specificity. These Abs were also detected in a fusion assay that showed that while they were a major component, they in fact represented only one-third to one-quarter of the total plasma neutralizing activity. A summary of the mapping data is shown in Fig. 10; it indicates that 8 of the 9 plasma samples contained at least 2 to 3 specificities that could be identified. However, in all cases, we could not account for all activity, suggesting that multiple nAb specificities of less breadth may also contribute to neutralizing activity.

Several other observations exemplify the complex nature of these plasma samples. (i) ConC gp120 adsorbed neutralizing activity against the matched isolate in all cases tested, yet only in 3 plasma samples was this truly cross-neutralizing. (ii) Plasma C1-0457 was effectively adsorbed using ConC gp120, which lacked a V2 loop, yet neutralization of JR-FL was knocked out by V2 loop mutations. (iii) Plasma C1-0763 was affected by mutants in V2 in a clade C background, but by V3 mutations and the N332A mutation on JR-FL. Part of this disparity is likely due to the backbone used to make these mutants, as clearly shown with N160 and N332 (Fig. 7 and 9), and thus, it is important to verify such findings in more than one background. A further caveat is that many Env point mutants are globally sensitive to distal specificities (Fig. 8 and 9). When this occurs, it suggests that the mutated residue is not

entirely solvent exposed and may be involved in trimer packing. This is in fact a very common phenomenon that has also been reported in several recent studies (9, 74, 78, 100, 101, 112). Our observation that a relatively larger fraction of gp41 MPER mutants than of gp120 mutants cause global sensitivity (compare Fig. 5B and C to Fig. 8 and 9) is consistent with the idea that the MPER is partially sequestered and is involved in protein-protein or membrane associations, so that substitutions often lead to global effects on folding. In contrast, most of our gp120 mutants are likely to be surface exposed and therefore to minimally affect trimer packing. A previous bioinformatics study identified a series of residues that partitioned with broad neutralization, i.e., polymorphisms at these positions were associated with the presence or absence of broad neutralization (34). Several of these residues corresponded to the bridging sheet and coreceptor binding site and were found to cause global sensitivity when mutated. Therefore, it is possible that the development of broad neutralization might relate to trimer "compactness."

In contrast to the findings of our study, Walker and colleagues found that the neutralization activity in 19 elite plasma samples was most often dominated by a single specificity and that MPER activity was minimal (112). It is not clear why the findings were different in the two studies. The Walker study used plasma from 14 subjects who ranked among the top 1% of neutralizers and from 5 additional subjects who ranked among the top 5% in a cohort of 1,798 subjects in International AIDS Vaccine Initiative (IAVI) protocol G (112). We used plasma from 9 subjects who ranked in the top 3% of a cohort of 308 subjects, including 3 subjects who were in the top 1%. The two studies also used different viruses and assay techniques. Previous studies have pointed out that detection of antibodies targeted to gp41 can depend on the target cells used in the assay. MAb 4E10 neutralization was found to be more sensitive in the TZM-bl assay (8), whereas other neutralizing antibodies directed to gp41 were better detected in the PBMC-based assay (30). These differences highlight the importance of examining multiple cohorts and using different approaches to capture the full spectrum of activities and to identify all possible targets on HIV-1 Env.

Various studies have hinted at the presence of unusual neutralizing specificities, some of which may be entirely novel. One report suggested that Abs overlapping the CCR5 binding site and sensitive to an I420R mutation in the bridging sheet can mediate broad neutralization (58). Although there is scant evidence for 2G12-like bnAbs from most mapping studies to date (4), recent studies revealed that a gp120 N332 mutation can sometimes ablate plasma neutralization, suggesting bnAb specificities that overlap the 2G12 epitope and may reside on the outer domain of gp120 (36, 38, 76, 109, 110).

In a recent review of mapping efforts to date, CD4bs activity accounted for a substantial proportion of the neutralizing activity in 12 of 43 plasma samples, whereas substantial MPER activity was present in 3 (4). Importantly, more than 50% of bnAb activity in 28 of the plasma samples could not be mapped. In approximately 50% of cases, gp120 failed to adsorb neutralizing activity (36, 37, 41). This suggests that much of the unmapped activity may target quaternary, trimer-dependent epitopes (35). Indeed, two recent studies (72, 112) have reported nAbs that recognize epitopes similar but not identical to those of MAbs PG9 and PG16. The results of another recent study suggested that the early nAbs preferentially target the CD4bs, while more broadly neutralizing Abs cannot be adsorbed by gp120 (i.e., PG-like or possibly MPER) and appear to be associated with CD4 T cell activation (66) and CD4 T cell decline upon infection (36).

Although MPER nAbs have been detected frequently in multiple studies, they only rarely exhibit neutralization breadth (4, 7, 37, 39, 41, 101). This may be because MPER nAbs tend to lack the potency of nAbs that target gp120. Thus, MPER neutralization may feature more prominently in plasma samples that exhibit somewhat weaker overall neutralization titers. It may therefore not be a coincidence that MPER accounted for little or none of the neutralization in our level 3 neutralizers (C1-0219, C1-0763, and C1-0457; top 1%) (findings similar to those of the IAVI study) but featured more prominently in at least 3 of our 6 level 2 neutralizers. The epitope of the MPER nAbs in these plasma samples resembled that of MAb 4E10, consistent with most previous studies (4). Despite its breadth, the lack of potency of 4E10 could explain why similar nAbs do not contribute significantly in most broadly neutralizing plasma samples. One possible reason why MPER nAbs are reported so commonly in mapping studies may simply relate to the fact that this is the only epitope to which we can measure neutralization directly, without the contribution of other nAbs that are present. With other epitopes, in contrast, mutant viruses, adsorption, and other techniques are needed in order to identify nAb activity against discontinuous conformational epitopes. Thus, we can detect exceedingly low titers of MPER nAbs, whereas at least half of the total neutralization activity must be affected to allow reliable measurement of other specificities using current technologies. In the case of plasma samples C1-0269, C1-0534, and C1-0536, this observation was consistent with MPER nAbs playing a significant role in the neutralization of most, but not all, viruses (Fig. 3C). Thus, the weak MPER activity detected in plasma samples C1-0175, C1-0219, and C1-0763 (Fig. 3A and B) is overshadowed by other nAbs targeted to gp120, as was suggested by the inability of MPER peptides to deplete neutralization of these plasma samples against most isolates tested (Fig. 3C and data not shown). An exception is C1-0175 neutralization of the Du156.12 isolate, which was largely explained by MPER nAbs (Fig. 3C). Thus, we can infer that other specificities contribute to the breadth of all 9 plasma samples, with MPER nAbs playing a supporting role in some, but not all, cases and that, in many cases, breadth is achieved by multiple specificities. Since we have identified several subjects whose samples naturally elicited MPER-neutralizing antibodies with some breadth, eliciting neutralizing MPER antibodies is a reasonable goal for HIV-1 vaccine design in concert with the elicitation of multiple broadly neutralizing specificities.

A substantial portion of ConC-neutralizing activity in the nine plasma samples was adsorbed by gp120 (Table 2), but only in the three level 3 neutralizers was cross-neutralizing activity against other viruses adsorbed. In the case of level 3 plasma sample C1-0763, V2-reactive activity was adsorbed by gp120, and the precise residues in V2 were mapped and shown to involve N160 and K169 (Fig. 6), which are also required by PG9 and PG16. Plasma C1-0219 was also found to contain V2 loop-reactive activity. This plasma sample was also sensitive to

the N160 and K169 mutations, suggesting epitopes related to those of the PG MAbs. The frequency of this specificity is consistent with that in the IAVI study, in which 5 of 19 plasma samples were N160 dependent. It may not be a coincidence that these two plasma samples were derived from clade A infections, as were the PG MAbs, raising the possibility that such specificities evolve more often during clade A infections. The potency of the V2 specificities is exemplified by the dramatic effects of N160 mutants on neutralization of the Q23.17 and CAP45 viruses by plasma samples C1-0219 and C1-0763. In each case, this V2 activity accounts for most, if not all, of the neutralizing activity that imparts extremely high ID_{50} titers, close to 10,000 (Fig. 2). It will be worthwhile to characterize this powerful activity more precisely in the future, especially if it cross-reacts with viruses from other clades.

Two recent plasma mapping studies further suggest that "PG-like" plasma nAbs may not precisely replicate the activities of the prototype MAbs (72, 112). Similarly, 2909-like quaternary nAbs isolated from SHIV-infected macaques exhibited specificities slightly different from that of the 2909 prototype (84). Taken together, these findings suggest that V2 or "PG-like" nAbs found in broad plasma samples can have various V-loop residue dependencies that may overlap but do not necessarily precisely replicate the known specificities. This is perhaps not surprising in view of the substantial variability of the V2 loop. The V2 dependencies of our three V2 specificity-containing plasma samples on ConC and JR-FL backgrounds are each substantially different, illustrating this point. One of these three plasma samples (C1-0457) was susceptible to a mutation at I165 of the JR-FL V2 loop (see Fig. S3 in the supplemental material) that was previously found to be a target of 5 plasma samples in the IAVI study, but distinct from PG-like nAbs, which are not sensitive to this mutation (112). This residue has been shown to regulate neutralization by trimer-specific MAbs 2909, 2.3E, and 2.2G (35, 84). Although these nAbs are strain specific, they recognize quaternary epitopes involving the V2 and V3 loops.

Three plasma samples (C1-0440, C1-0457, and C1-0763) were sensitive to the glycan at N332, depending on the virus strain. In at least four previous plasma mapping studies, this sensitivity has also been observed (76, 36, 112). One group has now isolated broadly neutralizing MAbs with this specificity via high-throughput screening of donor B cells (110). In our study, the neutralizing activity of at least one of our plasma samples, C1-0763, was broadly adsorbed by gp120 (Table 2), as observed for most of the previously reported N332-sensitive plasma samples (76, 112). However, there is a complexity of specificities in plasma C1-0763 (V2 linear, V3, MPER, N332A). Interestingly, unlike the N332A-dependent activity observed previously (112), plasma C1-0763 partially competed with biotinylated 2G12 for gp120 binding (Fig. 9B). It is worth noting that the N332 and N339 glycans are part of the alpha-2 helix, a known neutralizing epitope for clade C plasma samples (71, 85). Thus, it appears possible that, as with the V2 activity, broad neutralizing responses can ultimately develop from initially highly type specific responses, with time and sustained virus replication. Thus, N332A affects the neutralization by C1-0763 of clade B viruses (JR-FL and TRO.11) but not of the other viruses. This may be because this plasma is able to neutralize these other viruses via the V2 loop specificities discussed above, which may not cross-react with the clade B viruses.

C1-0219 is a clear example of multiple specificities in plasma mediating breadth. This plasma sample contained prominent neutralization activity directed to both the CD4bs and the V1V2 loops. Despite the breadth of the CD4bs nAbs, this activity did not explain all of the neutralizing activity against any of the isolates tested (Table 2). Indeed, the relative contribution of the CD4bs nAbs to the neutralization of a given virus appeared to depend on the overall sensitivity of the virus to the plasma sample. Thus, when C1-0219 exhibited modest neutralization titers (e.g., Du156.12 and SC422661.8), CD4bs nAbs played a major role (Table 2; Fig. 5A). However, when C1-0219 exhibited high neutralization titers, the contribution of CD4bs nAbs was less significant. Thus, while the partial adsorption seen with gp120 in this plasma was likely due to the D368R-sensitive CD4bs nAbs, the remaining activity was dependent on quaternary structures similar to those of the PG MAbs, which were sensitive to N160. Indeed, MAbs to both targets have now been isolated from this individual. These include antibodies CH01 to CH04, which map to the V2 region and are trimer dependent but are distinct from PG9/P16 (10), and three potent anti-CD4bs MAbs (113a) isolated using the RSC3 protein, as was done for VRC01 (114). Interestingly, isolates, such as CAP45.G3, that were only weakly neutralized by plasma CD4bs antibodies (Table 2) were very sensitive to the anti-V2 MAbs CH01 to CH04 (10), accounting for the potency of the plasma against this virus. Similarly, Du156.12 was sensitive to MAbs CH01 to -04 but not to the new anti-CD4bs MAbs. In contrast, isolates such as ZM109.4 were sensitive only to the anti-CD4bs MAbs, not to the anti-V2 MAbs isolated from C1-0219. These data confirm, through the use of monoclonal agents, that neutralization breadth can be mediated by two distinct antibody specificities in a single subject.

We were not able to determine the specificity of the cross-neutralizing activity in all the plasma samples. A case in point is that plasma C8-0258 was not significantly mapped by any method. This could be due to complexity of nAb specificities in this plasma or to our incomplete array of mapping tools. It is noteworthy that this individual was the only one infected with a clade B virus, and while the sample did show cross-clade neutralization (Fig. 2), it was weak against clade A. It is possible that breadth in this individual may be due to multiple narrow specificities or that we have overlooked CD4bs nAbs that are not D368R dependent (95), such as MAb HJ16 (18). Additionally, our study focuses on IgG-mediated neutralizing activities in the plasma and not on other antibody isotypes. We have found that purified IgA from the plasma of HIV-1^+ subjects can neutralize (data not shown), which indicates that IgA, in addition to IgG, may be responsible for some aspects of virus inhibition in both broad neutralizers and other HIV-1-infected subjects.

Overall, our results support the idea that multiple distinct bnAb responses can evolve in some people. In cases where neutralization appears to be more clonal, this may not rule out multiple specificities; it means only that one specificity dominates neutralization. Only with time do bnAb responses finally develop in some subjects, and there is evidence that ongoing viral replication contributes to the development of these bnAbs (36, 66, 89). What remains unknown is why only certain

individuals are capable of mounting a bnAb response. Is there something unique about the virus and the antigenic structure of its Env that favors antibody responses to conserved epitopes? The fact that the epitopes are present on most viruses would argue against this, unless regions outside the epitope can control immunogenicity. Another possible explanation is that the response is differentially controlled by host genetics. In this regard, our results suggest that the B cell response in certain individuals is privileged to target multiple unrelated bnAb epitopes that most likely require different IgG heavy and light chain gene usage. Identification of the biologic process governing these selective responses could yield valuable insights for vaccine design.

The significance of our findings also relates to the discovery of new neutralizing MAbs. In some instances, knowledge of the predominant nAb specificities may directly assist in MAb isolation. For example, in the present study, B cell receptor ligands consisting of parent CAP45.G3 gp120 and its K169E mutant might function effectively as positive and negative reagents for the selection of V2-neutralizing MAbs from the B cells of donor C1-0763 in a flow cytometry screening protocol similar to that reported recently (38, 114). Such rational approaches may accelerate the isolation of new bnMAbs, providing new tools for vaccine design.

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